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RESEARCH ARTICLE

Stem Cell Mesenchymal Injection Increases Platelet-Derived Growth Factors Level and Percentage of Collagen in Third-Degree Burn injured Mice

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ABSTRAK

Pendahuluan: *Mesenchymal stem cell (MSC)* terbukti mampu mempercepat penyembuhan luka. *Platelet Derived Growth Factor (PDGF)* dan kolagen mempunyai peran penting dalam penyembuhan luka. Namun, efek pemberian MSC terhadap kadar PDGF dan jumlah kolagen masih belum terungkap. **Tujuan:** untuk mengetahui pengaruh MSC terhadap peningkatan kadar PDGF dan prosentase jumlah kolagen pada penyembuhan luka bakar.

Metode: Penelitian eksperimental menggunakan 20 mencit BALB/C dibagi menjadi 4 kelompok yaitu: kelompok kontrol (G-0) diinjeksi aquadest 2 ml, kelompok 1 (G-1), kelompok 2 (G-2), dan kelompok 3 (G-3) masing-masing diinjeksi dengan MSC dosis 1 x 104, 2 x 104, dan 4 x 104.. LB dibuat dengan cara menempelkan paku yang telah dipanasi selama 20 detik di kaki tikus. Kadar PDGF hari ke 2 (PDGF-2) dan ke 7 (PDGF-7) diperiksa dengan metode ELISA, sedangkan hari ke 10 dilakukan eksisi kulit untuk menghitung prosentase jumlah kolagen.

Hasil: Terdapat perbedaan kadar PDGF-2, PDGF-7, dan persentase jumlah kolagen secara bermakna, $p < 0.05$. Analisis posthoc menunjukkan bahwa kadar PDGF-2, PDGF-7, dan prosentase jumlah kolagen pada G-2 dan G-3 lebih tinggi secara bermakna dibanding G-0, $p < 0.05$. Sedangkan kadar PDGF-2, PDGF-7, dan prosentase jumlah kolagen pada G-3 lebih tinggi secara bermakna dibanding G-2 dan G-1, $p < 0.05$.

Kesimpulan: Injeksi MSC dengan dosis 2 x 104, dan 4 x 104 pada LB dapat meningkatkan kadar PDGF dan prosentase kolagen.

Kata kunci: Mesenchymal stem cell, PDGF, kolagen.

ABSTRACT

Introduction: *Mesenchymal stem cell (MSC) has been demonstrated to accelerate wound healing. Platelet-derived growth factor (PDGF) and collagen have an important role in wound healing. However, the effect of MSC on levels of PDGF and number of collagen has not been established.* ***Objectives:*** *To determine the effect of MSC on the levels of PDGF and the percentage of collagen in the burns healing.*

Methods: *in this experimental study, 20 BALB /c mice were divided into 4 groups: a control group (G-0) injected with 2 ml of distilled water and group 1 (G-1), Group 2 (G-2), and group 3 (G- 3) injected with MSC at the dose of 1 x 104, 2 x 104, and 4 x 104 respectively. The burn wound was made by attaching a metal nail that had been heated for 20 seconds at the feet of mice. Levels of PDGF on day 2 (PDGF-2) and 7 (PDGF-7) was assessed by ELISA, while on day 10 excision of skin was done to calculate the percentage of collagen.*

Results: *There was a significant difference in the levels of PDGF-2, PDGF-7, and the percentage of collagen (P <0.05). Posthoc analysis showed that the levels of PDGF-2, PDGF-7, and percentage of collagen in the G-2 and G-3 was significantly higher than that of G-0, p <0.05. While the levels of PDGF-2, PDGF-7, and the percentage of collagen in the G-3 was significantly higher than that of G-2 and G-1 (p <0.05).*

Conclusion: *Injection of MSC at a dose of 2 x 1044 and 4 x 1044 increases the levels of PDGF and the percentage of collagen.*

Keywords: *Mesenchymal stem cells, PDGF, Collagen.*

INTRODUCTION

Burns are a tissue damage/loss of tissue due to a contact with the heat source including fire, hot water, chemicals, electrical current/lightning, and radiations. Burn degree can vary from first, second and third. The third degree burn is defined as wounds afecting the the epidermis, dermis and hypodermis of skin. Third burn degree is characterized by the white/pale color, hairless, scar, and no pain (De Jong, 2010). Wound will activate natural killer (NK) and macrophages then stimulates the growth of Platelet derived growth factor (PDGF), vascular epidermal growth factor (VEGF), Tumor growth factor β (TGF β), interleukin-1 β (IL-1 β) and fibroblasts. Furthermore, these growth factors trigger the formation of Matrix metalloproteinase (MMP), which then stimulates the formation of collagen (Kenji, 2010). The administration of mesenchymal stem cells at a dose of 0.5 × 106 in mice can accelerate the third burn degree

healing with a faster epithelialization. In addition, MSC was also been shown to increase levels of vascular Endothelial Growth Factor (VEGF), malondialdehyde (MDA) levels, and total tissue protein (Sukpat S, 2013). However, the effect of MSC on PDGF levels and the amount of collagen have not been established.

Burn incidence is high, especially household burn. Data from the Indonesian Ministry of Health in 2008 showed that the prevalence of burn in Indonesia accounted for 2.2%. Third burn degree requires a long time to heal, often leaving an aesthetic issue, and can also cause death. The mean number of burn patients treated in the Hospital Dr. Sardjito in a week every year was six people, most end in death. While the death rate from burn in the dr. Cipto Mangunkusumo Hospital in 2012 was 37% - 39% per year. Thus, more effective treatment for of burn especially third degree is needed. At present, some stem cell therapy in burns has been shown to result in a shorter recovery time, a low morbidity rate, and a minimal scaring risk. Burn treatment with stem cells is expected to heal burn faster, require a relatively short time, and leaves no scarring (Rodriguez, 2015). MSC for wound healing has been extensively studied. A study conducted by Ansari showed that MSC promotes wound healing characterized by an increase in the percentage of collagen (Ansari, 2013). Another study with third degree injured rats indicated that the MSC has been shown to increase levels of IL-1 (interleukin-1), IL-6 (interleukin-6), TNF-α (Tumor Necrosis Factor α), IL-10 (interleukin- 10), VEGF and collagen types I and III (Lingyin, 2014). However, the studies did not measure the levels of PDGF which has an important role in collagen formation and wound healing process (Karimi, 2014).

The beneficial effect of MSC on wound is thought to be directly through stimulating differentiation of the existing progenitor cells, or a paracrine effect. MSC paracrine effects of stem cells causing inactive hemopoetic to active, activates monocytes, which in turn increasing the number of macrophages in the network, as well as stimulating the formation of PDGF, TGF β and IL 1, which then activates the fibroblast (Amable, 2014). In addition, the MSC through transdifferentiation also increase the number of active fibroblasts, triggering the formation of collagen early and do not cause scarring (Yates, 2013). According to the International Society for Cellular Therapy (ISCT) mesenchymal stem cells can be obtained by isolating the umbilical cord of rats, then cultured and validated by three ways: i) checking whether the cells are able to attach to the plastic in standard culture conditions; ii) identifying marker cluster of differentiation (CD) 34, CD 105+ and CD 90+ by immunostaining; and iii) The ability to differentiate into bone and adipose cells vitro (Rantam, 2014).

This study was aimed to determine the effect of the injection of MSC on levels of PDGF and the percentage of collagen in third degree burns injured mice.

METHODS

This was an experimental study with posttest only control group design twenty BALB/c were divided into 4 groups consisting of: i. the control group (G-0), burn at the feet of mice was cleaned with distilled water and allowed to heal naturally; ii) Group 1 (G-1), around burn at the feet of mice were intramuscularly injected with MSC at the dose of 1x10 4; iii. Group 2 (G-2), MSC injected with a dose of 2x10 4 around burn affected area and intravenous..Group 3 (G-3), MSC injected intramuscularly with a dose of 4x10 4 around burn affected area (Li Wang, 2014). On day 2 and the 7, 0.2 ml blood sample were taken for PDGF evaluation. Blood were collected from orbital sinus using microhematocrit after anesthetized with ketamine dose of 4 mgr/kg. Blood was collected in the conical tube and centrifuged, PDGF monoclonal antibody supernatant were mixed, then screened by enzyme-linked immunosorbent assay (ELISA). On day 10, mice were anesthetized with ether, then sacrificed. Tissue were taken from the burn affected area (1x1x0.01 cm) embeded in paraffin preparations for a collagen evaluation. Preparations was stained using Masson's staining Trichrom (MT), and then observed under a light microscope at a magnification of 100 x.

The treatment were performed in the laboratory of Biology, Faculty of Biology, Semarang State University (UNNES). The histology assesment and reading results were conducted in the laboratory of Pathology Anatomy of medical Faculty, Sultan Agung Islamic University.

Preparation of Mesenchymal Stem Cell

MSC was isolated from the umbilical cord derived of mice, then the umbilical was cut into small pieces and placed in a Petri dish and isolated in a CO_2 incubator for 2 days, then harvested. Once harvested, cell surface markers were identified by flowcytometry protein marker CD 34--, CD 90+ dan CD 105+, The method used was Dissosiatic Enzimatic (Enzyme Collagenase) (SCCR Laboratory, 2015).

Preparation of Burn Injured Rats

Prior made burns, the fur around the feet was

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Table 1. Average level of PDGF-2, PDGF-7 and Percentage of collagen

Variables	Groups				
	G-0 (n=5; Σ ±SD)	G-1 (n=5; Σ±SD)	G-2 (n=5; Σ±SD)	G-3 (n=5; Σ±SD)	*P* (ANOVA)
BW (gram)	23.76 ±0.71	23.40 ±0.42	23.66 ±0.69	24.28 ±0.73	0.225
PDGF-2 (pg/mg)	0.046 ±0.004	0.065 ± 0.019	0.074 ±0.26	0.098 ±0.012	0.002
PDGF-7 (pg/mg)	0.049 ±0.005	0.068 ± 0.016	0,081 ±0,016	0.107 ±0.008	0.000
Σ Kolagen (%)	17.74 ±12.47	29.12 ±5.17	44.16 ±12.70	76.64 ±4.33	0.000

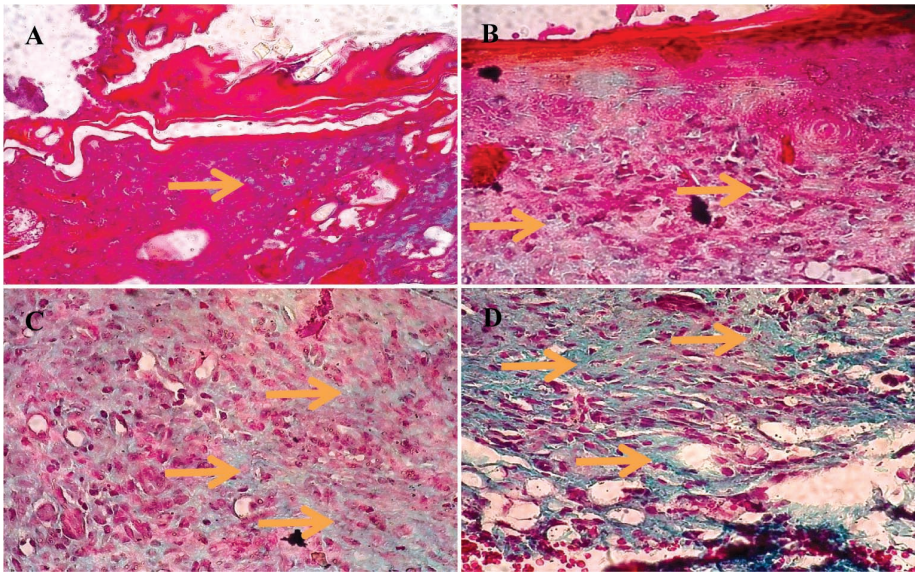

Figure 1. Collagen (bluish green) using Masson's Trichrome. A: G-0, B: G-1, C: G-2, and D: G-3.

shaved, then the mice were anesthetized with ether until the mice were seen fainting. The burns were made using the nail heads previously heated on fire to obtain the same depth of the wound (1x1x0.01 cm in width). Then the nail heads was attached to the skin of the legs of mice for 3 seconds until a third-degree burns were generated.

Statistical Analysis

The statistical analysis used in this study was ANOVA, followed by Post Hoc with the significance level of 95%. This study was conducted after the approval from the ethics committee medical faculty of Unissula Semarang was obtained.

RESULTS

The result of this present study showed that the levels of PDGF-2, PDGF-7, and the percentage of collagen is presented in table 1. While collagen is identified by the color of bluish green Masson's Trichrome staining (figure 1).

The highest Levels of PDGF-2 and PDGF-7, as well as the percentage of collagen was found in the G-3, followed by the G-2, G1, G-0. ANOVA test results showed that there were significant differences between groups ($p < 0.005$).

Levels of PDGF

Post hoc analysis of the results of the PDGF-2 showed that the level of PDGF in G-2 and G-3 were significantly higher than that of G-0 ($p < 0.05$ and $p < 0.001$ respectively). While the levels of PDGF-2 in G-3 was significantly higher than that of G-2 and G-1 ($p < 0.05$). The levels of PDGF-2 in G-1 and G-2 was not significantly different, $p > 0.05$. (figure 2).

Post hoc analysis results on the levels of PDGF-7 showed that the levels of PDGF-7 in G-1, G-2 and G-3 were significantly higher than that of G-0 ($p<0.05$). While the levels of PDGF-7 in G-3 was significantly higher than the that of G-1 and G-2 ($p < 0.005$). The levels of PDGF-7 on G-2 was not significantly different than the G-1, $p > 0.05$ (figure 2).

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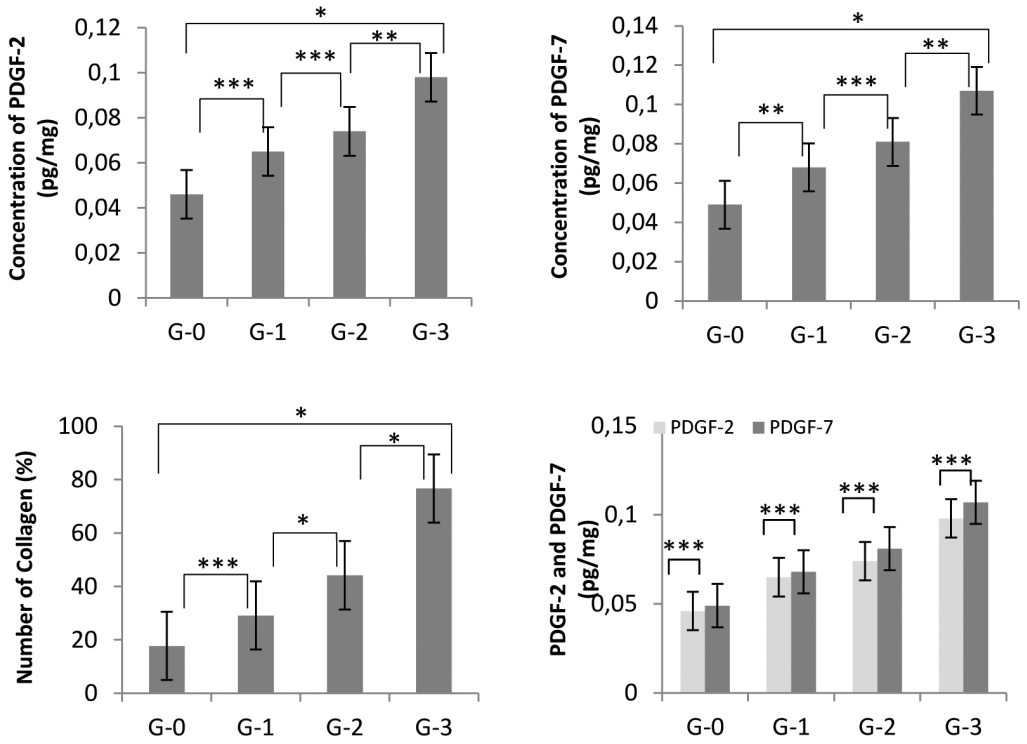

Figure 2. Concentration of PDGF-2, PDGF-7, and Number of Collagen, and Correlation between PDGF-7 and Number of Collagen. Note: * p < 0.001; ** p < 0.05; *** p > 0.05

The results of the analysis of independent T test showed that the levels of PDGF-7 were higher than the levels of PDGF-2 in all groups, but the difference was not significant (p> 0.05) (figure 2).

Number of Collagen

Post hoc analysis results showed that the percentage of collagen in G-1 was higher than that of G-0, but the difference was not statistically significant (p>0.07). The percentage amount of collagen in the G-2 and G-3 was significantly higher than that of G-0 (p<0.05). While the percentage of collagen in the G-3 was significantly higher than the G-1 and G-2, p<0.005. Similarly, the percentage of collagen in the G-2 compared to G-1 (p<0.05) (figure 2).

Correlation PDGF and The Percentage of Collagen

Pearson correlation analysis results showed a very possitive correlation between the levels of PDGF-7 and the percentage number of collagen, r = 0.873, p <0.001 (figure 3). This illustrates that elevated levels of PDGF-7 will be followed by an increase in the percentage of collagen.

DISCUSSION

These results indicated that administration of MSC at a dose of 1 x 104, 2 x 104, dan 4 x 104 has been shown to increase levels of PDGF on day 2 and day 7. The results of this study illustrates that the higher dose of MSC, the higher PDGF levels will be. It has not been established that the increase in duration of MSC will be followed by increased levels of PDGF because the results of this study showed no significant difference between the levels of PDGF on day one and the seven after the injection of MSC. Research reported by LingYin Liu (2014) showed that the MSC injection on wound in rats has been shown to increase levels of IL-10, VEGF, and PDGF after 3 weeks. Results of the study also showed that in the group receiving the injection of MSC had a ratio of collagen type I and III is higher than that of control. Evidences showed that during the wound healing process, the inflammatory phase, deposition of extracellular matrix (ECM) is formed, in which the synthesis of collagen propagated by growth factors and cytokines that PDGF, FGF, TGF β and IL-1 β produced by leukocytes and lymphocytes. Moreover, the process of tissue remodeling, growth factors such as PDGF, TGF β and IL 1 β, TNF α stimulates the synthesis of collagen and other connective tissue followed by cytokines and growth factors to modulate the synthesis and activation of metalloproteinases, enzymes that serve to relegation ECM components (De Jong, 2010).

The results also showed that the percentage of collagen or collagen density increased significantly

after injection of MSC. This is in line with research conducted by Karimi (2015) in experimental male BALB/c mice as many as 30 individuals divided into 3 groups: the control group, given the suspension and the group adipose stem cells. The results showed that more than five mice given stem cell had a significant epithelialization, the number of fibroblasts and collagen was higher compared to other groups. This was associated with the proliferative phase that occurs after the inflammatory phase i.e. on days 3-14. Proliferative phase is characterized by the formation of granulation tissue which is a combination of cellular elements, including fibroblasts and inflammatory cells, which in conjunction with the growth of new capillaries in the loose network of extra cellular matrix of collagen, fibronectin and hyaluronic acid. Fibroblasts that produce collagen in large quantities appears first day 3 and reached the peak on day 7. Collagen is a triple-chain glycoprotein, which is the main element of the extracellular matrix of wounds and useful to establish the strength of the scar tissue. Collagen was first detected on day 3 after injury and continued to increase until week 3 or within 3 months (Kuroda, 2013). While the maturation phase lasts from day 7 up to 1 year. Immediately after the extracellular matrix is formed, began the reorganization. This not only resulted in the migration of cells into the substratum and cell growth but also lead to a build up of collagen by fibroblasts (Romo, 2012). It shows that by day 10 after treatment there is a process of mesenchymal stem cells in wound healing by means of transdifferentiation through the Wnt signaling pathway spur of the number of active fibroblasts and triggers the formation of collagen (Karimi, 2015).

It can be concluded that the injection of MSC has been shown to fasten the healing process of burns compared with the conventional treatment and minimizes scarring, but it requires further researchs.

CONCLUSION

The injection of MSC at a dose of 1 x 104, 2 x 104, dan 4 x 104 can increase the levels of PDGF and the percentage of collagen in third degree burns in mice. In addition, there is a positive correlation between the levels of PDGF and the percentage of the amount of collagen.

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